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Lin, Sheng-Cai; Hardie, David

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AMPK – sensing glucose as well as cellular energy status

Sheng-Cai Lin¹ and D. Grahame Hardie²

¹State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University,

Xiang'an Campus, Xiamen, Fujian 361102, China

²Division of Cell Signalling & Immunology, School of Life Sciences, University of Dundee, DUNDEE, DD1 5EH, Scotland, UK

*Joint corresponding authors: Sheng-Cai Lin (linsc@xmu.edu.cn) and D. Grahame Hardie (lead contact) (d.g.hardie@dundee.ac.uk)

SUMMARY

Mammalian AMPK is known to be activated by falling cellular energy status, signalled by rising AMP:ATP/ADP:ATP ratios. We review recent information about how this occurs, but also discuss new studies suggesting that AMPK is able to sense glucose availability independently of changes in adenine nucleotides. The glycolytic intermediate fructose-1,6-bisphosphate (FBP) is sensed by aldolase, which binds to the v-ATPase on the lysosomal surface. In the absence of FBP interactions between aldolase and the v-ATPase are altered, allowing formation of an *AXIN-based AMPK activation complex* containing the v-ATPase, Ragulator, AXIN, LKB1 and AMPK, causing increased Thr172 phosphorylation and AMPK activation. This nutrient-sensing mechanism activates AMPK, but also primes it for further activation if cellular energy status subsequently falls. Glucose sensing at the lysosome, in which AMPK and other components of the activation complex act antagonistically with another key nutrient sensor, mTORC1, may have been one of the ancestral roles of AMPK.

Mammalian AMPK is usually regarded as a sensor of adenine nucleotides that is activated in states of low cellular energy, and acts to restore energy balance under those circumstances by switching on alternative catabolic pathways that generate ATP, while switching off anabolic pathways and other processes consuming ATP. This view of the system, which was proposed in the 1990s (Hardie and Carling, 1997), has prevailed for the last 20 years. However, recent findings (Zhang et al., 2017; Zhang et al., 2014; Zhang et al., 2013) show that mammalian AMPK can sense glucose in the absence of any changes in cellular energy state, and that this takes place on the lysosome where an AMPK-activation complex acts in opposition to another key sensor of cellular nutrient availability, the mechanistic target-of-rapamycin complex-1 (mTORC1). A major aim of this review is to discuss these new concepts, and to place them in context with the canonical energy-sensing role of AMPK. We will also speculate about the evolutionary origins of AMPK and its ability to sense cellular glucose availability and energy status. We will focus particularly on pathways upstream of AMPK rather than downstream events, since those have been reviewed extensively elsewhere (Day et al., 2017; Garcia and Shaw, 2017; Hardie et al., 2016).

Discovery of the energy-sensing role of AMPK in mammalian cells

Mammalian AMPK was originally discovered in independent studies as poorly characterized protein kinase activities derived from rat liver that phosphorylated and inactivated acetyl-CoA carboxylase (ACC) (Carlson and Kim, 1973) or 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Beg et al., 1973), the key regulatory enzymes of fatty acid and cholesterol synthesis respectively. The “ACC kinase” was found to be allosterically activated by 5'-AMP, and a farsighted proposal was made that this could be a mechanism to inhibit fatty acid synthesis (an ATP-consuming pathway) when cellular energy status was compromised (Yeh et al., 1980). Meanwhile, the “HMGR kinase” was shown to be part of a kinase cascade, being itself activated by phosphorylation by an upstream kinase (Ingebritsen et al., 1978). In 1987 the crucial observation was made that the ACC and HMGR kinase activities were functions of a single protein kinase that was activated both by AMP and by phosphorylation (Carling et al., 1987). Given the realization that AMPK had at least two downstream targets (a recent review has listed no less than 60 (Hardie et al., 2016)), it was renamed the AMP-activated protein kinase (AMPK) in 1988 (Hardie et al., 1989;

Munday et al., 1988). The critical activating phosphorylation site was subsequently identified to be Thr172, located in the catalytic subunit of rat AMPK (Hawley et al., 1996) (note that the exact residue numbering varies with species and isoform), while the principal kinase phosphorylating Thr172 was finally identified in 2003 [25 years after the initial observations by Ingebritsen et al (1978)] to be the tumor suppressor LKB1 (Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2003).

Role of AMPK orthologs in glucose-sensing in fungi and plants

Although their relationship with AMPK was not apparent until 1994 (Stapleton et al., 1994; Woods et al., 1994), genes in the budding yeast *Saccharomyces cerevisiae* that encode the AMPK ortholog were first described in 1977, with the isolation of *cat1* and *cat3* mutants that would not grow on glycerol or maltose (Entian and Zimmermann, 1982; Zimmermann et al., 1977), *ccr1* mutants that would not grow on ethanol (Ciriacy, 1977), and *snf1* and *snf4* mutants that would not grow on sucrose or raffinose (Carlson et al., 1981; Neigeborn and Carlson, 1984). The *cat1*, *ccr1* and *snf1* mutants were later shown to be alternate alleles of a single gene now termed *SNF1*, while the *cat3* and *snf4* mutants were alleles of a second gene now known as *SNF4*. The *SNF1* gene product had an N-terminal protein kinase domain essential for its function (Celenza and Carlson, 1986), while *SNF4* encoded a protein that at the time was of unknown function, although it was shown to form a complex with Snf1 and was required for Snf1 function (Celenza et al., 1989). Using yeast two hybrid screens, three other related gene products that interact with Snf1 (Sip1, Sip2 and Gal83) were later identified (Jiang and Carlson, 1997); although single knockouts of these had no effect, a triple knockout abolished Snf1 function (Schmidt and McCartney, 2000). Thus, Snf1 exists as a complex, which we term below the SNF1 complex, with Snf4 and one of Sip1, Sip2 and Gal83. Although it was initially claimed that the kinase activity of the SNF1 complex was not regulated by glucose (Celenza and Carlson, 1986) using more appropriate assays it was shown that its kinase activity increased dramatically upon glucose starvation (Wilson et al., 1996; Woods et al., 1994). Thus, the SNF1 complex is able to sense glucose availability.

Yeast strains with loss-of-function of the SNF1 complex are unable to grow on fermentable sugars other than glucose, because genes required for their metabolism (e.g. invertase, required for breakdown of sucrose) are not expressed in high glucose (*glucose repression*), and the SNF1 complex is required for their de-repression. Yeast grown in high glucose metabolise the sugar solely

by fermentation to ethanol (i.e. by glycolysis), because many genes required for oxidative metabolism are also subject to glucose repression and de-repressed by the SNF1 complex. The latter is therefore required to allow yeast to grow on sucrose or on non-fermentable carbon sources such as ethanol (hence the acronym *SNF* for sucrose, non-fermenting) (Hardie et al., 1998). When yeast cells are grown in batch culture in high glucose, they initially grow exponentially using fermentation, but once glucose starts to run out their growth temporarily ceases, then recommences after a lag period, a phenomenon known as the *diauxic shift*. The lag period represents, in part, the time required for derepression of genes involved in oxidative metabolism, with the latter allowing metabolism of ethanol and other non-fermentable carbon sources, although at the price of slower growth. In strains lacking *SNF1* or *SNF4*, the cells stop growing at or near the time of the diauxic shift, but do not recover because they fail to switch on oxidative metabolism (Haurie et al., 2003). Thus, the key role of the SNF1 complex in yeast appears to be in sensing of glucose availability (although it still remains unclear exactly how this is achieved), and in triggering the metabolic switch from glycolysis to mitochondrial oxidative metabolism when glucose runs low.

Intriguing evidence for a glucose-sensing role of AMPK orthologs in green plants came from studies of a primitive plant, the moss *Physcomitrella patens*. Plants with a knockout of both genes encoding orthologs of the catalytic subunits of AMPK grew normally if kept under 24 hr light, but died when grown in 12 hour light:12 hour dark cycles (Thelander et al., 2004). For a green plant, darkness is of course the equivalent of glucose starvation, because they can only make glucose from CO₂ by the reactions of the Calvin cycle when light is available.

Evolution of AMPK in early eukaryotes

It is now clear that AMPK exists universally as heterotrimeric complexes comprising catalytic α subunits and regulatory β and γ subunits (for example, in *S. cerevisiae* the α subunit is encoded by *SNF1*, the β subunits by *SIP1*, *SIP2* or *GAL83*, and the γ subunit by *SNF4*). Genes encoding these subunits are found in the genomes of essentially all eukaryotes (see next section for interesting exceptions), suggesting that the AMPK heterotrimer arose very early during eukaryotic evolution. One of the seminal events that led to the development of eukaryotic cells is considered to be the endosymbiotic engulfment of aerobic bacteria that eventually became mitochondria (Sagan, 1967).

Mitochondria have their own DNA and machinery for RNA and protein synthesis, and mitochondrial DNA still replicates independently of host cell nuclear DNA, even though most genes encoding mitochondrial proteins are now encoded within nuclear DNA. After the endosymbiotic event itself, and the host and endosymbiont also developed the ability to exchange ADP and ATP, it can be envisaged that the host cell would have needed a means of monitoring the primary output of the newly acquired endosymbionts, i.e. ATP, as well as a means to signal back to them when the provision of ATP was excessive or insufficient. Since AMPK monitors cellular adenine nucleotides, and the processes it regulates in modern day organisms include mitochondrial biogenesis (Zong et al., 2002), mitophagy (Egan et al., 2011) and mitochondrial fission (Toyama et al., 2016), one can argue that AMPK fits the bill to represent this ancient regulatory link between mitochondria and the host cell. Specifically, the ancestral AMPK might have been switched on by glucose deprivation, and then induced the more glucose-sparing mitochondrial oxidative metabolism (which generates >30 ATP per glucose) rather than the much more glucose-hungry glycolytic pathway (which generates just 2 ATP per glucose). As an example of this from modern day organisms, such a switch from glycolytic to oxidative metabolism occurs during the diauxic shift in budding yeast, where it requires the presence of the SNF1 complex, as discussed above.

Eukaryotes without AMPK – intracellular parasites

There are some interesting exceptions to the rule that all eukaryote genomes contain genes encoding the three subunits of AMPK, although these can be regarded as “exceptions that prove the rule”. Eukaryotes lacking AMPK found to date are all intracellular parasites that spend some or all of their life cycle living inside other eukaryotic cells, where the host cell provides nutrients and will also contain AMPK to maintain energy balance on behalf of the parasite. One example is *Encephalitozoon cuniculi*, a microsporidian parasite derived from fungi that infects mammalian cells, which is an opportunistic pathogen in immunocompromised humans such as those with AIDS. It has the smallest known eukaryotic genome encoding only 29 conventional protein kinases, and lacks genes encoding the α , β or γ subunits of AMPK (Miranda-Saavedra et al., 2007). *E. cuniculi* also lacks functional mitochondria that produce ATP, although it has mitochondrial remnants called *mitosomes* (Williams et al., 2008) as well as nuclear genes encoding proteins normally considered

mitochondrial (Katinka et al., 2001). Interestingly, *E. cuniculi* has four genes encoding ATP:ADP translocases related to those found in the chloroplasts of plants, and in the latter they export ATP into the cytoplasm in exchange for ADP. However, in *E. cuniculi* three of these are located in the plasma membrane, with the fourth in mitosomes (Tsaousis et al., 2008). Thus, this organism, which has no known extracellular form other than metabolically inert spores, may be able to survive without AMPK because it simply “steals” ATP from its host cell, which does express AMPK to maintain energy balance.

Other eukaryotes that lack genes encoding AMPK include *Plasmodium falciparum* and *P. berghei*, the causative agents of malaria in humans and rodents respectively (Miranda-Saavedra et al., 2012). However, related species that cause malaria in birds (*P. gallinaceum* and *P. relictum*) do have AMPK genes (Böhme et al., 2016), suggesting that *P. falciparum* and *P. berghei* may have lost them. Interestingly, *P. falciparum* and *P. berghei* have a gene encoding a protein kinase called KIN, which has a kinase domain closely related to that of AMPK. KIN has a threonine residue (Thr616) equivalent to Thr172 (Mancio-Silva et al., 2017), and a domain related to the autoinhibitory domain of AMPK (Fig. 1), suggesting that it might share some regulatory features with AMPK, although other regions of KIN and AMPK- γ are not related. KIN is not necessary for asexual reproduction of *P. berghei* in mice, which occurs primarily within erythrocytes, but is required for transmission of the sporozoite stage to the salivary glands of the mosquito (Tewari et al., 2010); it is therefore required for the extracellular but not the intracellular stages of the *P. berghei* life cycle. To summarise, it appears that *E. cuniculi* and *Plasmodium* species may be able to dispense with nutrient-sensing kinases when they are living inside host cells (which constantly provide nutrients and also contain AMPK), although species of *Plasmodium* may require such a kinase (either AMPK or KIN) when they are inhabiting an extracellular environment.

The canonical energy-sensing role of AMPK: regulation by adenine nucleotides

The tripartite mechanism of activation by AMP

ATP and ADP are the key components of the “rechargeable battery” of all living cells, with catabolism (or photosynthesis when present) “charging up the battery” by converting ADP to ATP, while most other cellular functions require energy and are driven by conversion of ATP back to

ADP, thus “flattening the battery”. Any increase in the cellular ADP:ATP ratio from its normal value of around 1:10 signifies an imbalance between the processes that generate and consume ATP. However, all eukaryotic cells also express adenylate kinase enzymes catalysing a reversible reaction ($2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$) that interconverts adenine nucleotides. If the adenylate kinase reaction operates close to equilibrium (as appears to be the case in most eukaryotic cells) then the AMP:ATP ratio will vary as the square of the ADP:ATP ratio (Hardie and Hawley, 2001), making changes in AMP a much more sensitive indicator of cellular energy status than changes in ADP or ATP (as first pointed out by Hans Krebs (Krebs, 1964)). Indeed, AMP is the key signal that activates AMPK, by three complementary mechanisms that are all opposed by ATP (Gowans et al., 2013): (i) promoting phosphorylation of Thr172; (ii) inhibiting dephosphorylation of Thr172; (iii) causing allosteric activation. This tripartite mechanism makes mammalian AMPK exquisitely sensitive to small changes in cellular AMP:ATP ratios (Hardie et al., 1999). Of the three components, mechanisms (i) (Oakhill et al., 2011) and (ii) (Xiao et al., 2011) but not (iii) (allosteric activation) have been reported to be mimicked by binding of ADP, although this usually requires up to 10-fold higher concentrations of ADP than AMP (Gowans et al., 2013; Ross et al., 2016a). Although the allosteric effect definitely contributes to AMPK activation in intact cells (Gowans et al., 2013) and occurs even with bacterially expressed AMPK that is not phosphorylated on Thr172 (Scott et al., 2014), AMPK activity is not significant in intact cells unless there is also some Thr172 phosphorylation (Willows et al., 2017b). It should also be noted that the allosteric mechanism appears to be absent in AMPK orthologs from budding yeast (Wilson et al., 1996) and plants (Crozet et al., 2014; Mackintosh et al., 1992).

Multiple isoforms of AMPK subunits

Mammals contain two genes (*PRKAA1*, *PRKAA2* in humans) encoding isoforms of the catalytic subunit ($\alpha 1$, $\alpha 2$ (Stapleton et al., 1996)), two (*PRKAB1*, *PRKAB2*) encoding isoforms of the β subunit ($\beta 1$, $\beta 2$ (Thornton et al., 1998)), and three (*PRKAG1*, *PRKAG2*, *PRKAG3*) encoding isoforms of the γ subunit ($\gamma 1$, $\gamma 2$, $\gamma 3$ (Cheung et al., 2000)). These could potentially give rise to up to twelve different heterotrimeric combinations, and all of those combinations can be generated by co-expression in insect cells (Myers et al., 2017). Although much remains to be learned about the roles of these different isoform combinations *in vivo*, there is already good evidence that they can

differ in their tissue distribution, regulation, subcellular localisation and function (Ross et al., 2016a; Ross et al., 2016b; Salt et al., 1998a). The $\alpha 2$ and $\alpha 3$ subunits in particular contain unique N-terminal regions of variable length that are absent from $\alpha 1$ and are unrelated to each other, and it seems likely that these are involved in targeting AMPK complexes to distinct subcellular locations (Pinter et al., 2013).

The molecular mechanisms underlying the canonical, tripartite mechanism by which mammalian AMPK is regulated by the adenine nucleotides AMP, ADP and ATP, which is at least partly understood due to the availability of several crystal structures, will now be discussed. We will start by describing the disposition of domains on the three subunits of the AMPK heterotrimer.

Structure and roles of the α subunits

The α subunits contain conventional serine/threonine kinase domains (α -KD) at their N-termini, containing the small N-terminal and larger C-terminal lobes (N- and C- lobes) typical of all protein kinases, with the catalytic site located between them (Figs. 1, 2). The C-lobe contains the activation loop with the conserved threonine residue (Thr172) whose phosphorylation is required for maximal activity (Hawley et al., 1996); Thr172 is phosphorylated *in vivo* by the tumor suppressor kinase LKB1 (Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2003) or the Ca^{2+} /calmodulin-independent kinase CaMKK2 (CaMKK α) (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). The kinase domains are followed by auto-inhibitory domains (α -AID), which received their name because constructs containing the α -KD and α -AID were about 10-fold less active than those containing the α -KD alone, even when both were maximally phosphorylated on Thr172 (Crute et al., 1998; Goransson et al., 2007; Pang et al., 2007). Crystal structures of isolated α -KD: α -AID constructs from *Schizosaccharomyces pombe* (Chen et al., 2009) and human $\alpha 1$ (Li et al., 2015) showed that, in these less active conformations, the α -AID binds to the “back side” of the α -KD

(i.e. opposite side to the catalytic site; see Fig. 2, lower panel left). The α -AID clamps the N- and C-lobes of the α -KD in an inactive conformation in which the residues of the “regulatory spine” (four non-consecutive hydrophobic residues that become aligned in the structures of protein kinases in active conformations (Taylor and Kornev, 2011)) are out of alignment. By contrast, in structures

of heterotrimers that are in active conformations with AMP bound to the α subunit (Calabrese et al., 2014; Li et al., 2015; Xiao et al., 2013), the α -AID has rotated away from this inhibitory position and now binds between the C-lobe and the α subunit instead (Fig. 2, upper panel left); the regulatory spine residues within the α -KD are also now aligned (Hardie et al., 2016).

The α -AID is connected to the C-terminal domain (α -CTD) by a region of extended polypeptide termed the “ α -linker” (Xiao et al., 2011; Xin et al., 2013). This plays a key role in the activation mechanism and is discussed further below. The α -CTD is a globular domain that terminates with a nuclear export sequence (NES) (Kazgan et al., 2010). Immediately preceding this NES is a serine/threonine rich loop (the ST loop) of about 50 residues that is not resolved in any of the existing crystal structures. This loop is present in AMPK orthologs from mammals and *Caenorhabditis elegans* but absent from those in *Drosophila melanogaster* and budding yeast (Hawley et al., 2014), and appears to represent a regulatory sequence that has been inserted during evolution. Phosphorylation of Thr496 (human numbering, NP_0062642) within the ST loop by Akt, in the $\alpha 1$ but not the $\alpha 2$ isoform (Hawley et al., 2014; Horman et al., 2006), and possibly by other kinases at the same (Hurley et al., 2006) or different (Suzuki et al., 2013) sites, regulates the phosphorylation and/or dephosphorylation of Thr172, thus allowing crosstalk between AMPK and other signalling pathways.

Structures and roles of the α subunits

The $\alpha 1$ and $\alpha 2$ subunits contain myristoylated N-terminal regions (Mitchelhill et al., 1997; Oakhill et al., 2010) followed by central carbohydrate-binding modules (α -CBM), α -linker regions, and then the C-terminal domains (α -CTD) (Figs. 1, 2). Only the α -CBM and α -CTD are fully resolved in existing crystal structures, so the structures of the myristoylated N-terminal regions and portions of the α -linkers remain uncertain. The α -CBM is a member of the CBM20 family of carbohydratebinding modules (Machovic and Janecek, 2006). Related domains occur in enzymes that metabolize starch or glycogen, such as branching and debranching enzymes, where they localize the associated catalytic domains on their polysaccharide substrates. The α -CBM does indeed cause a proportion of AMPK in cells to localize to glycogen particles (Hudson et al., 2003; Polekhina et al., 2003), via a binding site (Polekhina et al., 2005) that in Fig. 2 is occupied by a

model oligosaccharide, α -cyclodextrin. The physiological role of glycogen binding remains unclear, although it would colocalise AMPK with some of its physiological targets, i.e. the muscle (Jorgensen et al., 2004) and liver (Bultot et al., 2012) isoforms of glycogen synthase, and perhaps R5/PTG, a glycogen targeting subunit of protein phosphatase-1 (Vernia et al., 2009). It has also been suggested that this domain may allow AMPK to sense the status of cellular glycogen stores (McBride et al., 2009), which would be intriguing given its role in glucose-sensing discussed below, although more work is required to prove that idea. The α -CBM does have one other key function in that the cleft between it and the N-lobe of the α -KD is the binding site for allosteric activators exemplified by A-769662 (Cool et al., 2006) and 991 (Xiao et al., 2013), which will be discussed in more detail later.

Roles of the α subunits

The α subunits contain the binding sites for the regulatory nucleotides, AMP, ADP and ATP. Following the N-terminal regions that interact with the α -CTD, α subunit sequences contain four tandem repeats of a motif known as a CBS repeat, so-called because they are also found in the enzyme cystathionine β -synthase and invariably occur as tandem repeats (Bateman, 1997). In other proteins, which usually have just two repeats, they bind regulatory ligands containing adenosine, such as ATP or S-adenosyl methionine, in the cleft between the repeats (Ignoul and Eggermont, 2005; Scott et al., 2004). The four CBS repeats in the AMPK- α subunits form the shape of a flattened disc with one repeat in each quadrant, with four potential ligand-binding clefts coming together in the centre. However, despite the presence of four repeats, the AMPK- α subunits appear to bind a maximum of three molecules of adenine nucleotide. Because the nucleotide phosphate groups can bind to residues from more than one repeat, the three sites are named after the number of the repeat that contributes to binding of the adenine and ribose moieties of the nucleotide (Kemp et al., 2007). CBS2 is the unoccupied site, perhaps in part because a conserved aspartate that binds the ribose moieties of nucleotides in CBS1, CBS3 and CBS4 is replaced by an arginine in CBS2 (Xiao et al., 2007).

Intriguingly, in the structures of AMPK heterotrimers with AMP bound in all three sites, the α -linker can be seen to wrap around one face of the disc of the α subunit (Fig. 2, top panel), with

one short conserved sequence (α -regulatory subunit interacting motif-1, α -RIM1) interacting with the vacant CBS2 site, and a second (α -RIM2, also known as the α -hook) interacting with AMP bound in CBS3 (Chen et al., 2013; Xiao et al., 2011; Xin et al., 2013). It has been proposed that the binding of ATP rather than AMP in the CBS3 site would prevent this interaction (Xiao et al., 2011), which is supported by luminescence proximity assays measuring the interaction between fragments of AMPK containing the α -linker and the α subunit. As predicted by the model, the presence of AMP increased, whereas ATP decreased, the interaction between these constructs (Li et al., 2015).

Conformational changes during activation by AMP

The overall structure of the heterotrimer can be divided into two rather distinct regions: (i) the “catalytic module”, containing the α -KD, α -AID and α -CBM; and (ii) the “nucleotide-binding module”, containing the α -CTD, β -CTD, β subunit and part of the α -linker (see Fig. 2, bottom right). The α -linker can be viewed as a flexible “hinge” that connects these two modules, although the missing part of the α -linker that is not resolved in crystal structures will also play a role. It can easily be envisaged (Fig. 2, lower panel) that the release of the α -linker from the face of the β subunit containing the CBS3 site could cause the catalytic and nucleotide-binding modules to move apart. This might then have two effects: firstly, it would allow the α -AID to rotate back into its inhibitory position behind the α -KD (Chen et al., 2009; Li et al., 2015), explaining how AMP binding causes allosteric activation [mechanism (iii) above]; secondly, it would expose phosphoThr172, which in the active AMP-bound conformation lies in a deep, narrow cleft between the two modules, to dephosphorylation by protein phosphatases, thus explaining how AMP binding protects against dephosphorylation [mechanism (ii) above]. This model does not so readily explain why AMP binding promotes phosphorylation by upstream kinases [mechanism (i) above], but that will be addressed later.

Unfortunately, there are as yet no crystal structures of complete heterotrimers in inactive conformations to confirm this model, and it is important to note that the structure shown in the lower panel of Fig. 2 is partly conjectural. Nevertheless, there is experimental support for the model: (i) mutations in α -RIM1 or α -RIM2, or in β subunit residues that interact with them, abolish allosteric activation by AMP (Chen et al., 2013; Xin et al., 2013); (ii) small angle X-ray scattering studies suggest that the heterotrimer adopts a more compact conformation upon binding

of AMP (Riek et al., 2008); (iii) assessed by luminescence energy transfer between probes attached to the Ntermini of the α and β subunits in a complete heterotrimer, AMP binding caused the two probes to move closer together, while ATP binding caused them to move further apart (Li et al., 2015). These results are consistent with an increased separation of the catalytic and nucleotide-binding modules upon binding of ATP rather than AMP.

Roles of the three nucleotide-binding sites on the α subunits

Although this model identifies CBS3 as the critical adenine nucleotide-binding site, it leaves open the function of the CBS1 and CBS4 sites. CBS4 was originally proposed to be a “nonexchangeable” site that irreversibly bound AMP, because when ATP was soaked into crystals containing three molecules of bound AMP, it exchanged with AMP only at CBS1 and CBS3 and not CBS4 (Xiao et al., 2007). Although ATP binding can occur at CBS4 (Chen et al., 2012), this was achieved by crystallization of a heterotrimer containing N-terminally truncated α and β subunits in the presence of ATP and absence of AMP, a rather non-physiological situation. Results of binding studies with wild type heterotrimers and fluorescent nucleotide analogs were also compatible with just two AMP-binding sites in the α subunit, one of high affinity ($K_d \approx 2 \mu\text{M}$) and the other of lower affinity ($K_d = 80\text{--}240 \mu\text{M}$) (Gu et al., 2017; Xiao et al., 2011). Assuming that the CBS4 site is non-exchangeable, this leaves CBS1 and CBS3 as the two sites where exchangeable binding was being measured. Although it was originally proposed that CBS1 was the high affinity site (Xiao et al., 2011), a more recent study (Gu et al., 2017) came to the opposite conclusion. Mutations were made in an $\alpha 1\alpha 2\alpha 1$ heterotrimer that would prevent binding of nucleotides at the catalytic site and at combinations of one or two of the three α subunit sites, and nucleotide binding was then re-measured. Their main conclusions (Gu et al., 2017) were that: (i) the CBS3 site binds AMP ($K_d = 17 \mu\text{M}$) with a 9-fold higher affinity than ATP ($K_d = 150 \mu\text{M}$); (ii) by contrast, the CBS1 site binds ATP ($K_d = 80 \mu\text{M}$) with somewhat higher affinity than AMP ($K_d = 160 \mu\text{M}$). Thus, CBS3 is the high affinity site whose occupancy by AMP correlates with AMPK activation. However, studies of mutant complexes in which only two sites were available suggested that the binding of nucleotides at CBS1 and CBS4 collectively alters the conformation of the CBS3 site to enhance its preference for AMP over ATP, thus helping to explain how AMPK achieves the potentially difficult task of sensing small changes in AMP in the presence of much higher concentrations of ATP. In their model, the CBS1 site will usually be occupied by ATP and the

CBS4 site by AMP, and it is only at CBS3 where AMP (and presumably ADP) binds in competition with ATP (Gu et al., 2017).

AMPK activation via the ADaM site

As briefly discussed in the section on the α subunits above, some AMPK activators bind in a cleft located between the α -CBM and the N-lobe of the α -KD (Fig. 2, top left). Most are synthetic compounds (derived from high-throughput screens that searched for allosteric activators of AMPK) rather than natural products, but many people in the field suspect that there is a naturally occurring metabolite that binds there, hence the name Allosteric Drug and Metabolite (ADaM) site (Langendorf and Kemp, 2015). When unoccupied, the ADaM site is stabilized by interactions between the phosphorylated form of Ser108 in the α -CBM (discussed further below) and two conserved lysine residues in the N-lobe of the α -KD (Li et al., 2015). Interestingly, mutation of these two lysines not only prevented activation by the ADaM site activator A-769662, but also prevented increased Thr172 phosphorylation in cells treated with agents that increase cellular AMP, while not affecting allosteric activation (Ross et al., 2017). Thus, occupancy of the ADaM site by an unidentified ligand may be able to influence AMPK activation by agents acting through the canonical energy-sensing pathway.

At least eight ADaM site activators have now been described (Fig. 3) of which just one (salicylate (Hawley et al., 2012)) is a natural product, although derived from plants rather than animals. In the form of extract of willow bark, salicylate has been used as a medicine by humans since ancient times (Jeffreys, 2004). It is also the major *in vivo* breakdown product of the synthetic derivative aspirin (acetyl salicylate), and may mediate some effects of aspirin that are independent of inhibition of the classical target, cyclo-oxygenase (Steinberg et al., 2013).

While compounds that bind to the ADaM site provide some protection against Thr172 dephosphorylation (Goransson et al., 2007; Sanders et al., 2007a), they are primarily allosteric activators. Thus, they cause markedly increased phosphorylation of the AMPK target ACC in intact cells, while often causing only modest increases in Thr172 phosphorylation [e.g. (Willows et al., 2017b)]. Many of them (those in the left-hand column in Fig. 3) only significantly activate AMPK complexes containing the $\alpha 1$ isoform, while others (“pan- α activators”, in the right-hand column in Fig. 3) also activate $\alpha 2$ complexes, although at somewhat higher concentrations. This is important,

since only the pan- α activators such as MK-8722 and PF-739 lower blood glucose in animal models of type 2 diabetes, because to do this they must activate AMPK and thus stimulate glucose uptake in skeletal muscle, where $\alpha 1$ is not expressed (Cokorinos et al., 2017; Myers et al., 2017).

The detailed molecular mechanism by which compounds binding to the ADaM site cause allosteric activation remains unclear. However, an α -helix in the α -linker (which is partially resolved in crystal structures with ADaM site activators bound (Xiao et al., 2013)), interacts with the C-helix in the N-lobe of the α -KD, and the conformation of the C-helix is known to be critical for the activity of other kinases (Taylor and Kornev, 2011). Interestingly, “naïve” AMPK heterotrimers that are not phosphorylated on Thr172, while being up to 1000-fold less active than the phosphorylated forms, are subject to much greater allosteric activation by A-769662 (>65-fold (Scott et al., 2014) as opposed to <5-fold) with phosphorylated heterotrimers (Cool et al., 2006; Goransson et al., 2007)). This appears to be because binding of ADaM site activators partially mimics the effect of Thr172 phosphorylation on the conformation of the α -KD activation loop (Willows et al., 2017b). Despite this, phosphorylation of ACC in cells lacking both LKB1 and CaMKK2 was negligible, even after simultaneous treatment with agents that bind to the ADaM site and that increase cellular AMP (Willows et al., 2017b), emphasizing the importance of Thr172 phosphorylation to AMPK activity in intact cells.

Binding of some (Sanders et al., 2007a) but not all (Willows et al., 2017a) ADaM site activators requires prior phosphorylation of Ser108. Although originally reported to be an autophosphorylation site (Scott et al., 2014), other kinases may phosphorylate Ser108 *in vivo*, so that other signalling pathways may be able to modulate the response of AMPK to the currently unidentified ligand(s) that bind the ADaM site. Identification of this ligand, if indeed it exists, remains a major challenge for the future.

The non-canonical glucose-sensing role of AMPK: the lysosomal pathway

Association of AMPK with membranes

The $\alpha 1$ and $\alpha 2$ subunits of AMPK are N-myristoylated (Mitchelhill et al., 1997; Oakhill et al., 2010), and this is necessary for activation of AMPK by glucose deprivation as well as association of

AMPK with cellular membranes (Oakhill et al., 2010; Zhang et al., 2017). In COS7 cells in which GFP-tagged $\alpha 1$ was co-expressed with $\alpha 1$ and $\beta 1$, glucose deprivation caused the diffuse cytoplasmic fluorescence to switch to a speckled, perinuclear pattern that was resistant to the detergent digitonin; this change was not observed using a non-myristoylatable mutant (G2A) of $\alpha 1$ (Oakhill et al., 2010). When AMPK was immunoprecipitated from COS7 cells, Thr172 phosphorylation by upstream kinases was also stimulated by AMP using wild type $\alpha 1$, but not the G2A mutant (Oakhill et al., 2010). Stimulation of Thr172 phosphorylation and activation by AMP, using LKB1 as the upstream kinase, has also been observed with AMPK immunoprecipitated from HEK-293 cells (Ross et al., 2016a), or biochemically purified from rat liver (Gowans et al., 2013), but not with AMPK expressed in bacteria (Sanders et al., 2007b) which, unlike the other preparations, would not have been N-myristoylated. Thus, stimulation by AMP of the phosphorylation and activation of AMPK by LKB1 might require prior association with membranes under physiological conditions.

AXIN is a scaffold tethering LKB1 to AMPK

In a search for novel physiological roles of AXIN, originally discovered as a component of Wnt signaling whose deficiency leads to duplication of the body axis (Zeng et al., 1997), an adenoviral vector carrying an shRNA designed to silence AXIN was injected into the tail vein of mice. Unexpectedly, triacylglycerols accumulated in the liver, particularly after overnight starvation, suggesting a role for AXIN in fat metabolism (Zhang et al., 2013). It was suspected that compromised activation of AMPK might be playing a role, and AXIN knockdown indeed reduced Thr172 phosphorylation under basal conditions, after glucose starvation, and after treatment with the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Co-precipitation studies suggested that AXIN bound LKB1 constitutively, but that AMP (although not ADP) promoted the interaction between AXIN and AMPK. Thus, formation of a ternary complex between AXIN, AMPK and LKB1 was greatly enhanced in isolated hepatocytes deprived of glucose or treated with AICAR, while complex formation in cell-free assays was enhanced by AMP (Zhang et al., 2013). These findings suggested that AMP binding to AMPK enhanced its binding to the AXIN-LKB1 complex, thus promoting Thr172 phosphorylation and potentially explaining component (i) of the tripartite mechanism of AMPK activation by AMP described above. However,

at that time the role of membrane association was not appreciated, which is perhaps why high concentrations of AMP (>200 μ M) were required for formation of the ternary complex with AXIN.

Activation of AMPK occurs on the lysosomal surface

The missing link between N-myristoylation and membrane association, and the formation of the AXIN:LKB1:AMPK complex, was finally identified the following year (Zhang et al., 2014). A yeast two-hybrid screen searching for new AXIN-interacting proteins identified p18/LAMTOR1, a protein that is anchored to the lysosomal membrane by N-terminal myristoyl and palmitoyl modifications (Nada et al., 2009). p18/LAMTOR1 plays a central role in the activation of the mechanistic target-of-rapamycin complex-1 (mTORC1) in response to nutrients such as amino acids and glucose (Bar-Peled et al., 2012; Sancak et al., 2010). It is a component of the Ragulator, a pentameric complex that interacts with RagA/RagB, alternative partners in the heterodimeric RagGTPase complex with RagC/RagD. In response to availability of certain amino acids, the Ragulator converts RagA/RagB to their active GTP-bound forms, triggering translocation of mTORC1 to the lysosome where it is further activated by the GTP-bound form of another G protein, Rheb. This translocation is mediated by Raptor, a key subunit of the mTORC1 complex, which binds to RagA/RagB:GTP (Saxton and Sabatini, 2017). As well as interacting with Rags, the Ragulator also forms a “super-complex” with the vacuolar ATPase (v-ATPase), the multisubunit proton pump that hydrolyses ATP to provide the energy to acidify the lumen of the lysosome (Marshansky et al., 2014). Indeed, a functional v-ATPase is required for the mTORC1 pathway to sense availability of amino acids and glucose (Zoncu et al., 2011).

Returning to the role of p18/LAMTOR1 in the LKB1-AMPK pathway (Zhang et al., 2014), its knockdown in the liver using siRNA was found to block AMPK activation in response to starvation *in vivo*. This effect could also be reproduced *in vitro*, since in p18/LAMTOR1-deficient mouse embryo fibroblasts (MEFs), AMPK was no longer activated upon glucose removal. AMPK activation was also abolished by knocking down the v0c subunit of the v-ATPase, suggesting that the v-ATPase was also required, while the association of AXIN, LKB1, AMPK, LAMTOR1 and the v-ATPase was increased upon glucose starvation. Taken together with results from immunofluorescence microscopy and subcellular fractionation assays, it was concluded that the AXIN:LKB1 complex translocates to the lysosomal surface under glucose deprivation conditions. Interestingly, by subcellular fractionation some AMPK was detected on the lysosomal surface even

before glucose starvation (Zhang et al., 2014), most likely due to N-myristoylation of the α subunits. After translocation, AXIN forms a new “super-complex” consisting of the v-ATPase, Ragulator, LKB1 and AMPK (note that each of the four latter sub-complexes themselves have multiple subunits, i.e. at least 14, 5, 3 and 3 respectively). We refer to this super-complex as the *AXIN-based AMPK-activation complex*, in which LKB1 phosphorylates and activates AMPK. Indeed, knockout of AXIN abolishes formation of this complex, as well as phosphorylation and activation of AMPK, in response to glucose starvation (Zhang et al., 2014).

The roles of the v-ATPase and the Ragulator in activation of AMPK were further analyzed. The v-ATPase:Ragulator complex provides docking sites for translocation of the AXIN:LKB1 complex to the lysosome, since genetic disruption of either caused a failure of formation of the AXIN-based AMPK-activation complex on the lysosomal surface (Zhang et al., 2017; Zhang et al., 2014).

Consistently, membrane fractions containing the v-ATPase:Ragulator complex [“light organelles”, prepared as in Zoncu et al (2011)] showed a higher affinity for the AXIN:LKB1 complex when purified from MEFs starved of glucose rather than those in full medium. The activity of the vATPase was also involved, because addition of the v-ATPase inhibitor concanamycin A increased the interaction between the v-ATPase and Ragulator, and promoted the translocation of AXIN:LKB1 to the lysosome, thus mimicking the effects of glucose deprivation. Intriguingly, upon binding to the v-ATPase-Ragulator complex, AXIN, which is an intrinsically disordered scaffold protein able to form multiple intramolecular loops (Luo et al., 2005; Oldfield and Dunker, 2014), undergoes conformational changes as shown by findings that the N-terminal fragment of AXIN exhibited a higher affinity for v-ATPase and Ragulator (Zhang et al., 2014). These results define a route, referred to as the lysosomal pathway, by which glucose deprivation is linked to AMPK activation.

Glucose sensing does not require any change in adenine nucleotide ratios

Although these findings suggested that inhibition of the v-ATPase primed the formation of the AXIN-based AMPK-activation complex, how glucose deprivation was linked to the v-ATPase and to the lysosomal pathway remained unclear. Collaborative work between our two groups has now mapped the detailed chain of events leading from glucose starvation to AMPK activation (Zhang et al., 2017). Although it has been known for many years that depriving cells of glucose activates

AMPK (Salt et al., 1998b), it had generally been assumed that this occurs because reduced glucose catabolism leads to ATP depletion, thus activating AMPK via the canonical energy-sensing mechanism described earlier. Unexpectedly, however, in MEFs that were transferred from high glucose to medium containing any glucose concentrations below about 5 mM, AMPK was activated without any changes in cellular AMP:ATP or ADP:ATP ratios (Zhang et al., 2017). Similar findings were made in livers of mice starved overnight, when blood glucose dropped from 9 to 3 mM, suggesting that this mechanism is relevant under physiological conditions *in vivo*. We also demonstrated that the AMP:ADP independent mechanism for glucose sensing, unlike the canonical energy-sensing mechanism, required AXIN and LAMTOR1 as well as N-myristoylation of the AMPK- α subunits, with glucose deprivation promoting translocation of AMPK from the cytoplasm to the lysosome upon glucose deprivation. This mechanism operates with AMPK complexes containing either α or β subunit isoform (Zhang et al., 2017). Interestingly, however, C-terminal farnesylation of LKB1 was not required for glucose sensing, although it was required for canonical energy-sensing by AMPK, as reported previously (Houde et al., 2014).

The glucose-sensing mechanism

How is glucose actually sensed by this lysosomal pathway? We have shown that aldolase, as well being as a glycolytic enzyme, is a sensor for glucose availability that regulates AMPK. When aldolase is unoccupied by its substrate fructose-1,6-bisphosphate (FBP), whose levels rapidly decrease upon glucose deprivation, the formation of the AXIN-based AMPK-activation complex is promoted. Evidence supporting this model includes, firstly, cell-free reconstitution assays showing that addition of FBP led to dissociation of the AXIN:LKB1 complex from the v-ATPase: Ragulator complex in light organelle fractions from glucose-starved MEFs. Secondly, knockdown of aldolases activated AMPK even in cells provided with abundant glucose. Thirdly, perhaps the most convincing evidence came from expression of the D34S mutant of aldolase, which has an almost unchanged K_m for FBP despite a greatly reduced K_{cat} (Morris and Tolan, 1993). FBP will therefore accumulate in the active site of this mutant, even when the flux through glycolysis is very low, mimicking the effect that high glucose has on the wild type. Consistent with our model, expression of this mutant caused a complete failure to activate AMPK upon glucose removal (Zhang et al., 2017).

Interplay between the energy-sensing and glucose-sensing mechanisms

Although glucose deprivation activated AMPK in MEFs without any changes in adenine nucleotides, in HEK-293 cells there were small increases in AMP:ATP or ADP:ATP under the same conditions (Zhang et al., 2017); this difference may be due to different dependencies of these two cell lines on glycolysis for ATP production. Tellingly, however, although the effect was smaller than with the wild type kinase, glucose deprivation of HEK-293 cells still activated AMPK that had a mutation (R531G) that renders the kinase insensitive to changes in AMP or ADP (Hawley et al., 2010). Returning to MEFs, although glucose deprivation did not cause any change in adenine nucleotides for up to 2 hours, this appears to be because the cells still had access to 4 mM glutamine and 1 mM pyruvate from the medium. If the glutamine was removed as well as glucose, after a lag of about 30 minutes (which may be the time required to metabolize the pyruvate) there was a much larger activation of AMPK that did correlate with large increases in AMP:ATP or ADP:ATP ratios (Zhang et al., 2017). In this scenario, it seems that AMPK is initially activated through phosphorylation via LKB1 at the lysosome in an AMP-independent manner, and that activation is then further enhanced through binding of AMP. Here, AMPK is acting as a dual sensor, firstly as a glucose sensor for fuel surveillance, and then as an adenine nucleotide sensor that monitors subsequent falls in energy status. Thus, the glucose-sensing and energy-sensing pathways of AMPK activation can operate independently, but if both are active they reinforce each other. Consistent with this, the interaction between AXIN and AMPK in a light organelle fraction from glucosestarved MEFs, where AXIN is already associated with the v-ATPase and Ragulator, was stimulated by as little as 5 μ M AMP, although if the light organelles were derived from control MEFs higher concentrations of AMP were required (200 μ M) (Zhang et al., 2014). However, glucose-sensing does not appear to require binding of AMP at the high-affinity CBS3 site on AMPK, because AMPK activation still occurred with the R531G mutant of α 2 (Zhang et al., 2017), whose affinity for binding of AMP at the CBS3 site is reduced by one to two orders of magnitude (Scott et al., 2004).

An evolutionarily conserved glucose-sensing mechanism?

Thus, our recent studies have defined a novel AMP/ADP-independent mechanism by which glucose starvation triggers activation of AMPK in mammalian cells, via sensing of the absence of FBP in the active site of aldolase (Fig. 4). Intriguingly, aldolases from budding yeast and mammals (Fba1

and ALDOA/B/C respectively) have been previously shown to physically interact with various subunits of the v-ATPase, and even to be required for assembly and activity of the v-ATPase complex (Lu et al., 2007; Lu et al., 2001; Lu et al., 2004). Even more interestingly, the association between aldolase and FBP in yeast was reported to increase dramatically when glucose was present in the medium, which led to the proposal that aldolase was a sensor of glucose availability, albeit at that time only thought to be involved in regulation of the v-ATPase (Lu et al., 2004).

Since there is good evidence that a major role of the yeast and plant AMPK orthologs is also in glucose sensing (see above), this leads to the interesting possibility that sensing of glucose, rather than energy, may have been one of the ancestral roles of the AMPK system. Indeed, it has always been puzzling that neither the yeast SNF1 complex (Wilson et al., 1996) nor the higher plant ortholog (termed SNF1-related kinase-1, SnRK1 (Crozet et al., 2014; Mackintosh et al., 1992)) are allosterically activated by AMP. This was especially puzzling given that glucose starvation of yeast caused activation of the SNF1 complex due to decreased dephosphorylation at the site equivalent to Thr172 (Rubenstein et al., 2008), and that this was associated with large increases in ADP:ATP and AMP:ATP ratios (Wilson et al., 1996). There is in fact some evidence that the dephosphorylation of the SNF1 and SnRK1 complexes at the sites equivalent to Thr172 are inhibited by binding of ADP (Mayer et al., 2011) or AMP (Sugden et al., 1999) respectively. However, even if these observations are correct, this means that the yeast and plant orthologs are only modulated by one of the three mechanisms by which mammalian AMPK is regulated by adenine nucleotides. It seems possible that sensing of glucose was the ancestral role of the AMPK system in the early eukaryote, and that energy sensing is a refinement that came later and has reached its most sophisticated form with the tripartite mechanism observed in mammals. Having said that, some components of the mammalian glucose-sensing pathway are not conserved in other eukaryotes. While aldolase, the vATPase, AMPK, and LKB1-related upstream kinases (albeit only related within the kinase domain) appear to be universal, AXIN is not found in a recognizable form in lower eukaryotes. Also, although there is a functional equivalent of the Ragulator in *S. cerevisiae* (the EGO complex), the individual subunits show limited sequence similarity with their counterparts in mammals (Powis et al., 2015). Thus, the roles of AXIN and the Ragulator may have been added later as a refinement of the mechanism for sensing glucose in multicellular eukaryotes.

Why is fructose-1,6-bisphosphate the key signal, and the lysosome the location for activation?

FBP may be an ideal signal to indicate flux through glycolysis because it is the product of the first irreversible step in the pathway, catalyzed by 6-phosphofructo-1-kinase. Once formed, FBP is largely committed to glycolysis, although a proportion of downstream flux can be used for serine and glycine synthesis. FBP is also well known as a feed-forward allosteric activator of the L, R and M2 isoforms of pyruvate kinase, thus promoting the final step in glycolysis (beyond the branch to serine and glycine) when glucose is abundant (Jurica et al., 1998). In bacteria, FBP also activates ADP-glucose pyrophosphorylase and hence glycogen synthesis (Dietzler et al., 1974;

Ribereau-Gayon et al., 1971), although this appears not to be the case with the equivalent enzyme in mammals (UDP-glucose pyrophosphorylase), where glycogen synthesis may be regulated by phosphorylation of glycogen synthase by AMPK instead (Bultot et al., 2012; Jorgensen et al., 2004).

Aldolase may be an ideal sensor because its K_m for FBP ($>40 \mu\text{M}$) is higher than the cellular concentration of FBP observed even with high glucose in the medium ($\approx 10 \mu\text{M}$). Aldolase is therefore unlikely ever to become saturated with FBP, so that the availability of FBP could be sensed across the entire physiological range. Aldolase would function as a surveillance system, sensing a fall in glucose available for catabolism even before any fall in cellular energy status had occurred, and activating AMPK in an AMP/ADP-independent manner, thus protecting cells from potential energy stress. Oxidative metabolism, including fatty acid oxidation and mitochondrial biogenesis, are major pathways switched on by AMPK, and these would provide alternative mechanisms to generate ATP. Thus, this mechanism would prepare the cells or organisms for conditions of low glucose availability, by switching on glucose-sparing oxidative pathways as opposed to the glucose-hungry pathway of glycolysis. In addition, activated AMPK would turn off the synthesis of fatty acids and cholesterol by inactivating ACC and HMGCR, the prototypical substrates of AMPK.

Another interesting question concerns why the lysosome is the site of AMPK activation during glucose starvation. One speculation comes from the fact that many simple single-celled eukaryotes, such as amoebae, primarily feed by engulfing food by *phagocytosis* or *pinocytosis*, with the resulting vesicles being delivered to the lysosome for digestion. Moreover, all eukaryotic cells carry out *autophagy* to engulf their own cellular contents and deliver them to lysosomes for digestion, especially during periods of starvation. Lysosomes can therefore be regarded as the

digestive systems or “gut” of the single cell, so that it might be appropriate to sense nutrient availability there.

Interplay between the mTORC1 and AMPK signaling pathways

It is intriguing that the lysosome is also the location where the mTORC1 complex is activated by the availability of amino acids and glucose (Saxton and Sabatini, 2017). There is evidence that, once activated, mTORC1 may subsequently dissociate from the lysosome in an active form (Manifava et al., 2016), although the mechanism by which it remains active is unclear. The new findings concerning the lysosomal pathway for AMPK activation emphasize the close connections between the regulation of the mTORC1 and AMPK systems, which may both be ancient nutrientsensing pathways that arose very early during eukaryotic evolution. AMPK phosphorylates the TSC2 component of the TSC1:TSC2 complex (Inoki et al., 2003), which carries a GTPase activating protein (GAP) function for the mTORC1-activating G protein, Rheb. This leads to inactivation of mTORC1 function in intact cells, although the exact molecular mechanism remains unclear. However, AMPK activation can still inactivate mTORC1 even in TSC2-deficient cells, which led to the discovery that AMPK also phosphorylates Raptor (Gwinn et al., 2008). Although once again the detailed mechanism for mTORC1 inactivation by Raptor phosphorylation is unclear, Raptor is the component of the mTORC1 complex that binds to the Rag heterodimer bearing the appropriate combinations of GTP and GDP (Sancak et al., 2008). Since phosphorylation of Raptor by AMPK induces binding of 14-3-3 proteins (Gwinn et al., 2008), it seems possible that this may prevent the translocation of inactive mTORC1 to the lysosome, and hence mTORC1 activation. Despite these multiple and apparently redundant mechanisms by which AMPK inactivates mTORC1, other studies revealed that metformin and phenformin (AMPK activators that work by inhibiting the mitochondrial respiratory chain (Hawley et al., 2010)), as well as glucose starvation, still suppress mTORC1 even in TSC2-null and AMPK-null MEFs by causing dissociation of mTORC1 from the lysosome (Efeyan et al., 2013; Kalender et al., 2010; Zhang et al., 2014). Mutations of RagA/B that abolish the GTPase activity, also completely abrogate inhibition of mTORC1 by glucose starvation, suggesting that Rags may play a more direct role in controlling mTORC1 in response to nutrients (Efeyan et al., 2013; Kalender et al., 2010). The newly identified glucose sensor, aldolase, becomes a new player in this scenario. Knockout of AXIN in MEFs led to prolonged activation and much slower dissociation of mTORC1 from the

lysosome after glucose starvation, most likely caused by the ability of AXIN to inhibit the GEF activity of the Ragulator complex, suggesting a direct role of AXIN in mTORC1 regulation (Zhang et al., 2014). The ability of AXIN to negatively regulate mTORC1 may also account for some of the beneficial effects of metformin (Zhang et al., 2016). These new findings suggest that mTORC1 can be regulated on different levels, through different sets of molecules. It can also be suggested that the modulatory role of AMPK may be secondary to direct dissociation of mTORC1 from the lysosome caused during AMPK activation on the lysosomal surface. It is also interesting to note that the connection between glycolysis and mTORC1 may be broader than anticipated. For example, it has been reported that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) physically associates with Rheb, inhibiting the interaction between mTORC1 and Rheb and hence mTORC1 activation. However, occupancy of GAPDH by its substrate glyceraldehyde-3-phosphate prevents this interaction and thus activates mTORC1 (Lee et al., 2009).

Conclusions and perspectives

Genes encoding the subunits of the AMPK heterotrimer occur in all eukaryotes, with the exception of a few intracellular parasites that spend most of their life cycle inside other eukaryotic cells, which may have been able to dispense with AMPK because their host cell already has it. The canonical role of AMPK is in monitoring cellular energy status by sensing the levels of AMP and ADP relative to ATP, and it can be speculated that this evolved in the early eukaryote to monitor the output of the newly acquired bacterial endosymbionts that developed into mitochondria. The mammalian AMPK complex is a complex molecular machine that achieves the difficult task of detecting changes in the concentrations of AMP and ADP in the presence of much higher concentrations of ATP. AMPK is also activated by glucose deprivation by a non-canonical mechanism involving the formation at the surface of the lysosome of the “AXIN-based AMPK activation complex” involving the v-ATPase, p18/LAMTOR1, Axin, LKB1 and AMPK. This mechanism can occur in the absence of any changes in AMP:ATP or ADP:ATP ratios, although the energy-sensing and glucose-sensing pathways can also operate together and reinforce each other. Glucose sensing involves the binding of the glycolytic intermediate fructose-1,6-bisphosphate to the glycolytic enzyme aldolase, which inhibits the formation of the AXIN-based AMPK-activation complex. It is interesting to speculate about which mechanism of AMPK regulation came first, i.e. energy-sensing or glucose-sensing, but both probably arose during early

eukaryotic evolution. Finally, the discovery of the novel glucose-sensing pathway raises many new questions. For example, does AMPK dissociate from the lysosome following its activation to fulfill its many roles in regulating cell function? Is the N-myristoylation of the AMPK- α subunit regulated, and does it cause AMPK to associate with membranes other than lysosomes? Is it aldolase occupied by FBP that represses AMPK activation by LKB1 at the lysosome, or is it unoccupied aldolase that activates phosphorylation of AMPK by LKB1? Are there additional player(s) that regulate the configuration of FBP-unoccupied aldolase in complex with v-ATPase, which seems to ultimately govern AXIN translocation onto the lysosome?

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FIGURE LEGENDS

Figure 1: Sequence similarity between the human AMPK- α 1 subunit and KIN from *Plasmodium falciparum*, and disposition of domains on typical mammalian AMPK- α , - β and - γ subunits. The top panel shows a dot plot comparison of the sequences of human AMPK- α 1 (entire sequence, residues 1-550) and *P. falciparum* KIN (residues 381-765). A dot is plotted if the sequence comparison scored >27 on the BLOSUM62 scoring matrix using a window size of 8 residues (default settings on CLC Main Workbench). Regions of similarity are revealed as diagonal lines highlighted by ovals, and can be seen to occur within the kinase domain and, less obviously, the autoinhibitory domain (α -AID) of AMPK. Note that there are no sequences equivalent to the α -linker or α -CTD in KIN. The first 380 residues of KIN, which show no similarity with AMPK, have been omitted. The lower panel shows the disposition of the domains on typical α , β and γ subunits of AMPK (see text for details); the domain map of the α subunit is aligned with the dot plot at the top.

Figure 2: Two views of the structure of the active form of the human α 1 β 2 γ 1 complex, with three molecules of AMP bound and phosphorylated on Thr172 (top), and a hypothetical structure of the inactive form with ATP bound (bottom). The structure at the top is from PDB file 4RER (Li et al., 2015), and is viewed from two orientations rotated 70° about the y axis. The structure at the bottom, viewed from the same orientations, is a hypothetical inactive conformation made by combining the α -KD and α -AID from PDB file 4RED (Li et al., 2015) and the remaining domains from 4RER, to illustrate potential relative movements of the α -KD and α -AID, and of the catalytic and nucleotide-binding modules, compared with the active state. Both models were created using “sphere” representation of C, N and O atoms in PyMOL v1.7.4.2 for Mac OSX, with the different domains color-coded and labelled.

Figure 3: Structures of allosteric activators of AMPK that bind in the ADaM site. Compounds in column (A) are selective for complexes containing the α 1 isoform, whereas those in column (B) are pan- α activators that activate both α 1 and α 2 complexes.

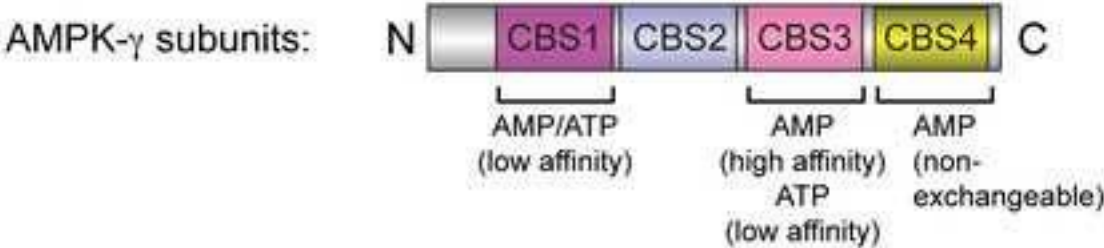
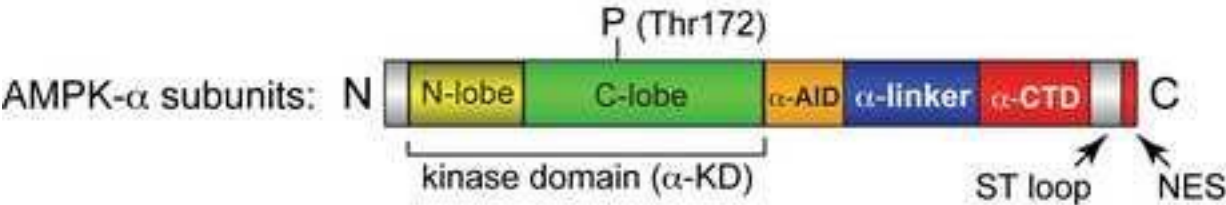
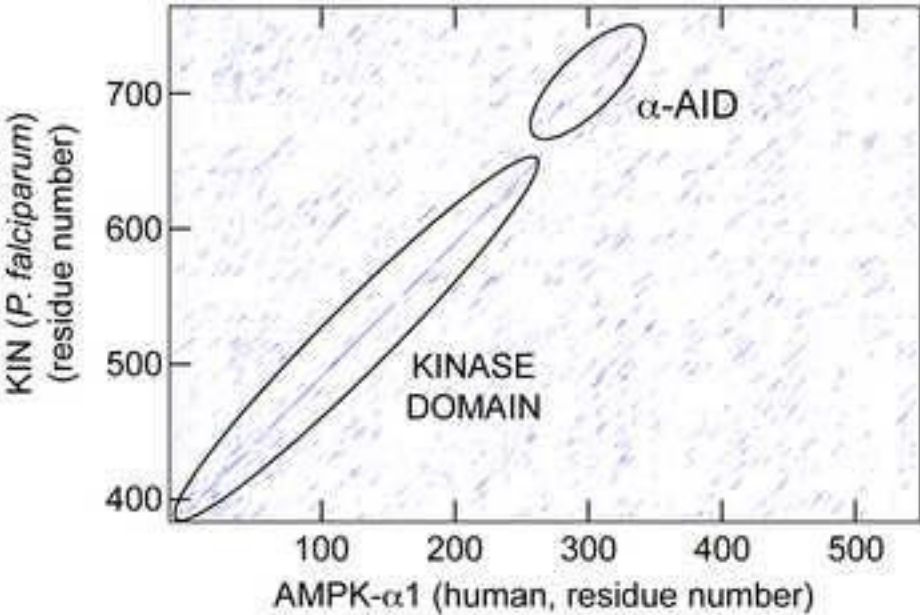
Figure 4: Schematic diagram depicting model for glucose sensing and regulation of AMPK

and mTORC1 on the surface of lysosome. In high glucose, aldolase is occupied by FBP, and the Rag:GEF activity of the Ragulator converts RagA/B to their GTP-bound form, recruiting mTORC1 to the lysosome where it may be activated if other conditions are appropriate (RagC/D in GDP form, Rheb in GTP form). In low glucose, dissociation of FBP from aldolase causes a change in its interaction with the v-ATPase:Ragulator complex, causing dissociation of mTORC1 and the formation of a “super-complex” involving the v-ATPase, Ragulator, AXIN, LKB1, and AMPK (the *AXIN-based AMPK activation complex*), thus triggering phosphorylation of Thr172 on AMPK. At least a portion of AMPK may already be on the lysosome due to the myristoylation of its α subunit. Note that the Ragulator is also anchored to the lysosome by lipid modifications (myristoylation and palmitoylation). See text for details.

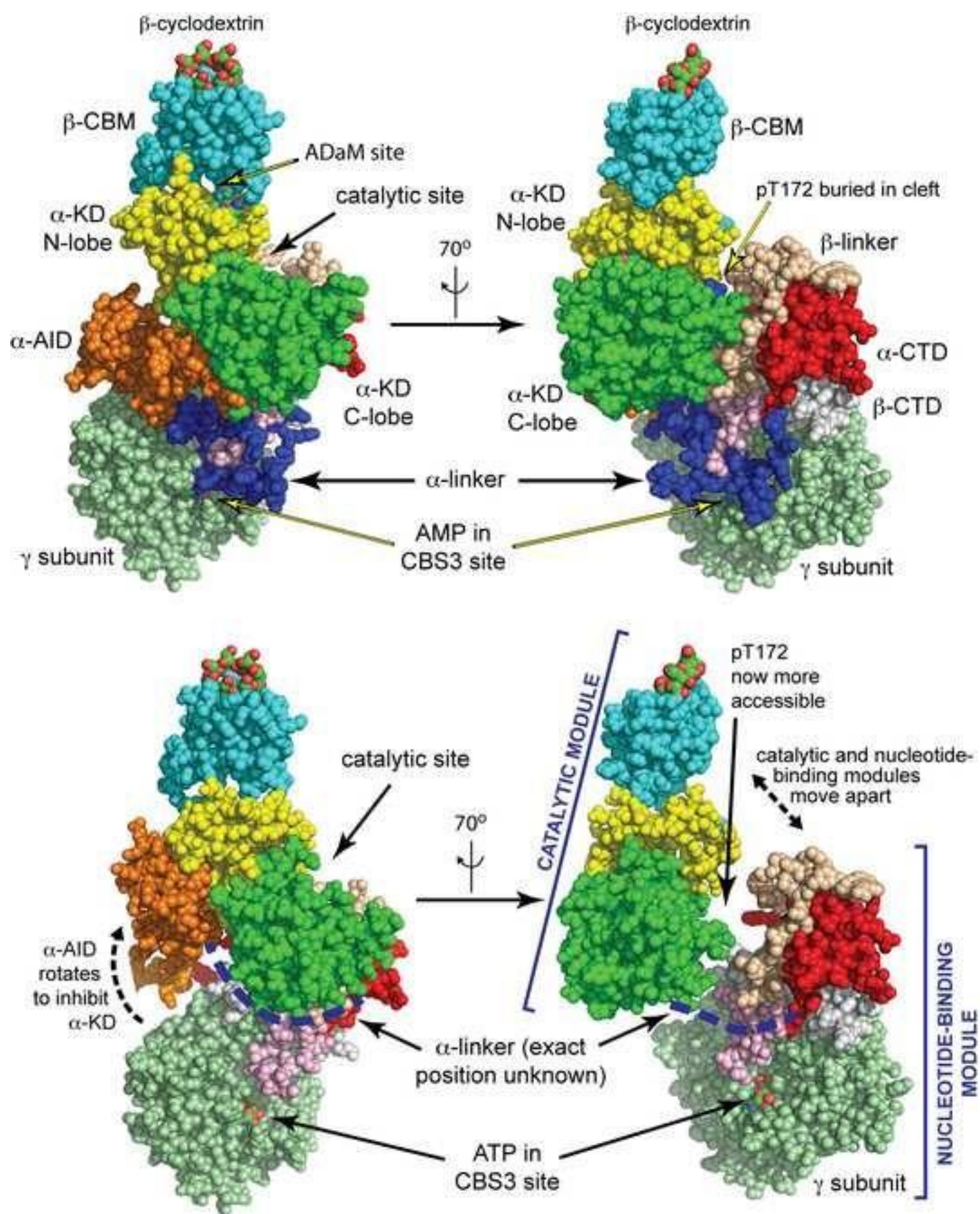
Figure 5: Regulation of mTORC1 by glucose availability.

The activity of mTORC1 is regulated by two parallel mechanisms. In response to glucose depletion, the GEF activity of Ragulator towards RAG is suppressed, converting RAG to the GDP-loaded form and switching off mTORC1. In addition, AXIN associates with the Ragulator, inhibiting its GEF activity towards RAGs. Activated AMPK can also phosphorylate TSC2 and activate its GAP activity to inactivate Rheb, or directly phosphorylate Raptor, a key component of mTORC1, leading to the inhibition of mTORC1.

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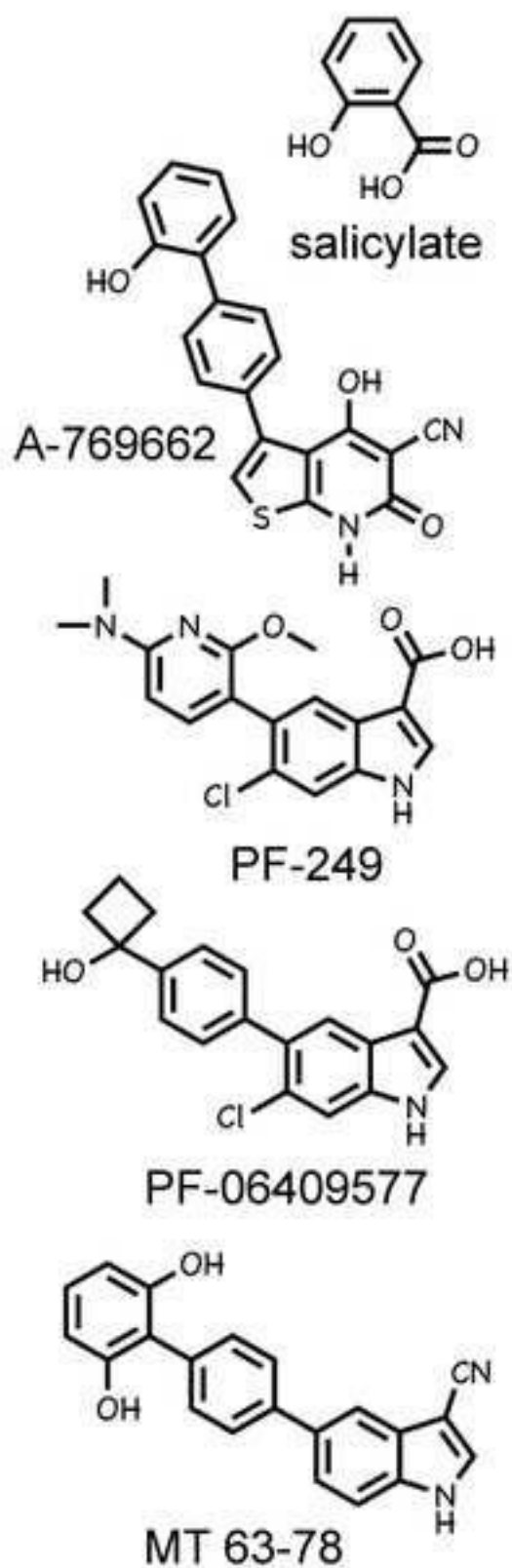


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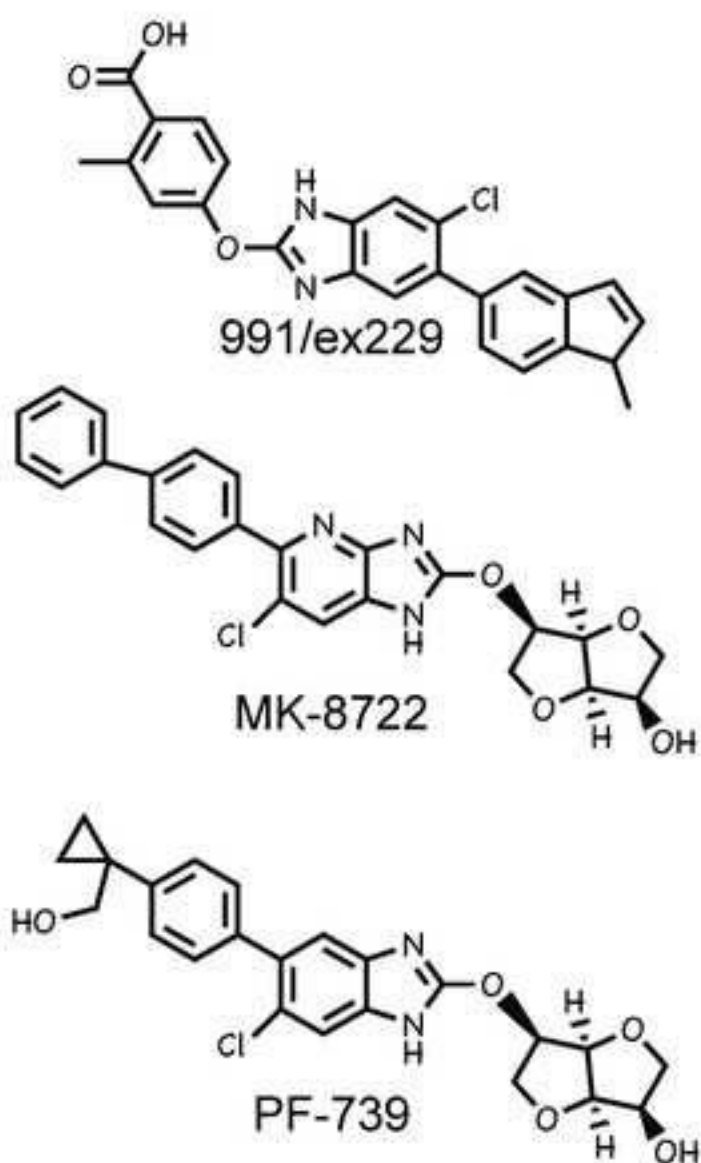


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A) β 1-selective activators:

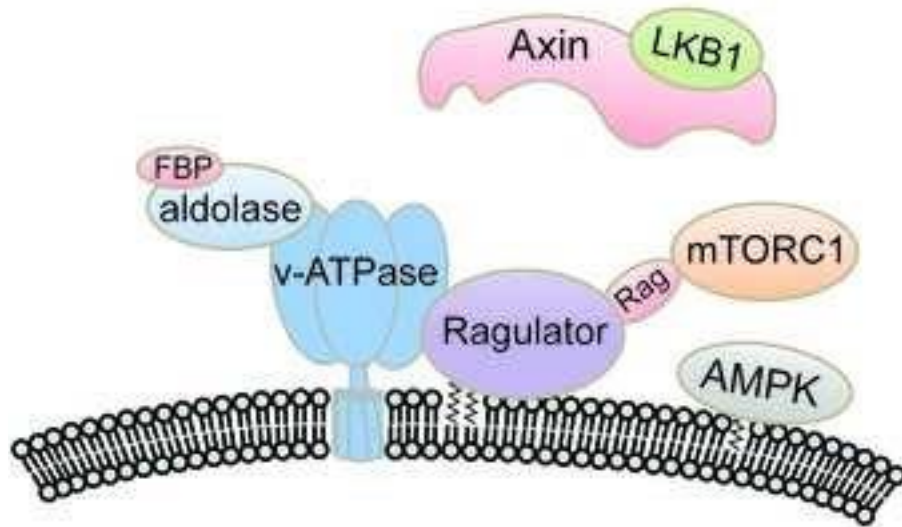


B) pan- β activators:

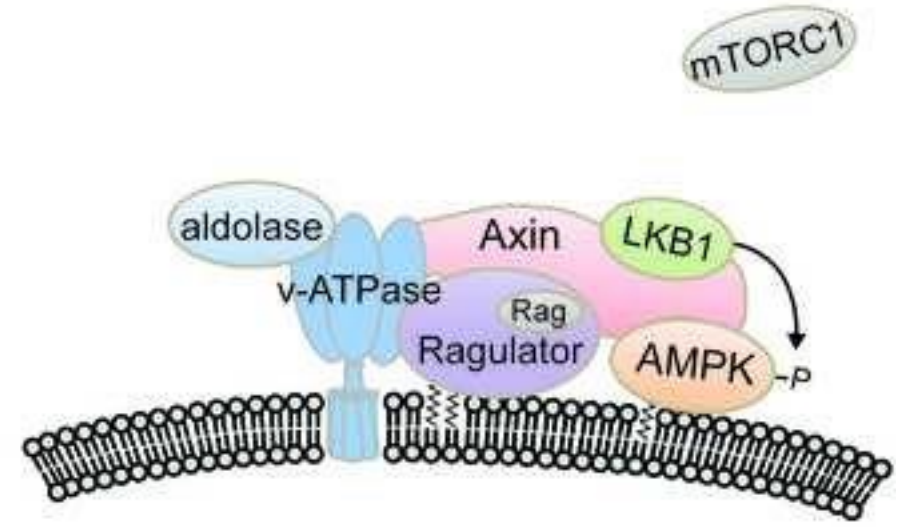


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